Induction of redox sensitive extracellular phenolics during plant–bacterial interactions

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Physiological and Molecular Plant Pathology 66 (2005) 90–98

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Induction of redox sensitive extracellular phenolics during plant–bacterial interactions☆

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Accepted 9 May 2005

Abstract

This study focuses on the transient and complex nature of phenolics that accumulate in the extracellular environment of plant suspension cells during the first few hours of the interaction between these plant cells and bacterial pathogens. Using suspension cells of *Nicotiana tabacum* we identified four acetophenones and four hydroxycinnamic acid amides that accumulate in this extracellular environment. Treatment of the suspension cells with isolates of the plant pathogen *Pseudomonas syringae* or heat-killed bacteria increased elicitation of extracellular phenolics and changed the composition of the compounds that accumulated. These phenolics were sensitive to oxidative stress; when suspension cells were treated with bacterial strains or elicitors that triggered an oxidative burst, these phenolics were oxidized and depleted for the duration of the burst. The qualitative and quantitative makeup of phenolics produced by *N. tabacum* suspensions was also affected by plant cell age and density. To our knowledge, this is the first study that closely follows the kinetics of individual extracellular phenolic compounds and the concurrent oxidative stress during the first few hours of a plant–bacterial interaction.
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Keywords: Antioxidant capacity; Oxidative stress; *Pseudomonas syringae*; *Nicotiana tabacum*; Caffeoylputrescine; Feruloylputrescine; Hydroxyacetosyringone; Hydroxyacetovanillone; Acetosyringone; Feruloyltyramine

One of the unique aspects of plants is their ability to produce a broad variety of phenolic compounds. Known roles for these phenolics include phytoalexins for disease defense, lignin production for structural strength, and antioxidants to counter prooxidants produced during pathogen or environmental stresses [10,23,29]. In human and animal physiology, plant phenolics have been found to have a wide range of beneficial effects, which has led to a renaissance in the search and study of bioactive plant phenolics [17,24,25,27]. Most of the medicinal properties attributed to plant extracts, such as anti-depression activity, anti-tumor activity and cardio-health, are linked to bioactive phenolics. For example, the plant phenolic acetosyringone has been shown to inhibit inflammation and asthma in humans through inhibition of both NADPH oxidases and actin polymerization in granulocytes [22]. Phenolics from *Artemis vulgaris* (mugwort) inhibit monoamine oxidase in the brain and can reduce depression [14]. It is becoming apparent that the physiological properties of phenolic metabolites goes beyond their 'antioxidative' characteristic.

A new role for phenolic compounds in plants is becoming evident especially relating to plant–microbe interactions. The animal studies mentioned above demonstrate that the bioactivity of phenolics can be due to the recognition of their unique and specific chemical structure

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0885-5765/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.pmpp.2005.05.002

by regulatory mechanisms. There are also several examples of this role for phenolics in plants. Acetosyringone, a phenolic produced at wound sites in plants, triggers *vir* gene transcription in the wound pathogen, *Agrobacterium*, allowing transfer of T-DNA to host cells [15]. In roots of nitrogen fixing plants, specific flavonoids activate *nod* genes in *Rhizobium* that are essential for establishing symbiosis [26,33]. In leaves, certain phenolic glucosides serve as signals that induce production of syringomycin toxin by *Pseudomonas syringae* pv. *syringae* [20]. These examples demonstrate that as intimate host–microbe associations evolved, phenolic metabolites were often selected to provide critical regulatory signals for the interaction.

With the exception of a few specific examples, the role of phenolics in the regulation of early plant–pathogen interactions has not been systematically examined. We have reported that extracellular phenolic antioxidants accumulated rapidly in suspension cells and attenuated the oxidative burst that often accompanies resistant plant–bacterial interactions [7]. Further identification of these compounds revealed that they were not the typical phenolics normally associated with lignin synthesis, but that they belonged to groups of phenolics associated with bioactivity in both plants and animals. We recently reported that one of these phenolics, acetosyringone, was able to influence the timing of plant–bacterial recognition responses [6]. Because these extracellular plant phenolics accumulate in contact with bacterial pathogens and have been associated with bioactivity, we felt it was important to examine their role in host–pathogen interactions more critically. Here, in this first step, we report the qualitative and quantitative makeup of the individual phenolics during the first hours of the plant–pathogen or plant–elicitor interaction. We also demonstrate their redox sensitivity and examine some parameters that affect their accumulation.

1. Materials and methods

1.1. Chemicals

Horseradish peroxidase (P-8250), guaiacol (G-5502), and luminol and all other chemicals were purchased from Sigma–Aldrich Chemicals, Inc. (St Louis, MO), unless otherwise noted.

1.2. Plant material

Suspension cells of tobacco (*Nicotiana tabacum* L. cv. Hicks) were maintained and prepared as previously described [5]. Routinely, 2-day-old tobacco cultures were used for assays. Cells were washed and suspended in assay buffer, containing 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 175 mM mannitol and 0.5 mM MES (pH 6). Unless otherwise noted, the cell density was 0.05 g ml^{-1}. Tobacco cell suspensions, 25 ml, in 50 ml beakers, were equilibrated for 0.5 h in a rotary water bath shaker set at 27 °C and 180 rpm for tobacco cells. Treatments were added directly to the suspensions. The viability of tobacco cells remained constant during all experiments described as estimated by Evans Blue staining [4]. All experiments were preformed at least twice with two or more replicates per treatment.

1.3. Bacterial preparations

Cultures of *Pseudomonas syringae* pv. *syringae* 61 were maintained and prepared as previously described [5]. Isolate WT (HR+) causes a hypersensitive reaction when infiltrated into tobacco. Isolate B7 (HR−) is a Tn5 insertion mutant that does not induce a hypersensitive response [2]. Bacterial cultures were grown for 20 h in Kings B broth, centrifuged, washed and suspended in deionized water. Based on optical density, the concentration of the suspension was adjusted with water so that addition of about 200 μL of the bacterial suspension to plant cell suspensions would result in the desired final concentration of bacteria, routinely 10^7 cfu ml^{-1}, unless otherwise noted. Bacterial concentrations in cell suspensions were verified periodically by dilution plating. Heat-killed preparations of isolate WT (HR+) were prepared as above and then autoclaved 20 min prior to treating cell suspensions.

1.4. HPLC-UV quantification phenolics

One-milliter samples of tobacco cell suspensions were filtered through Miracloth and centrifuged at 12,000*g* for 5 min prior to HPLC analysis. When not analyzed immediately, samples were flushed with N_2 and stored overnight at −20 °C. Phenolics were separated by C_{18} RP-HPLC using a Waters (Milford, MA) quaternary pump, autosampler, photodiode array detector, and Empower data acquisition on a Dell Pentium 4 computer. A 250×4.6 mm i.d., 5 μm Luna C18(2) analytical column (Phenomenex, Torrence, CA) was used with a binary mobile phase gradient of methanol in 0.01% aqueous phosphoric acid as previously described [32]. Aliquots, 100 μL, of samples were acidified with phosphoric acid (0.1%) and placed in the autosampler using a 30 μL injection volume. Quantification of peak area was preformed using the UV_{max} wavelength for each peak and reported as relative HPLC units.

1.5. Identification of phenolics

Atmospheric pressure ionization mass spectrometry analysis was performed on a Quattro LC benchtop triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) using the electrospray ionization interface in the negative mode (ES^-) as previously described [32]. Mass spectrometric data were acquired in the full scan mode over the *m*/*z* 50–400 range. Sensitivity of the mass spectrometer was optimized using an acetosyringone standard. A Waters 2690 HPLC system using the same column and gradient as

described for HPLC-UV analysis was utilized for separation of the phenolics. Samples of tobacco suspensions for mass spectroscopy were prepared as for HPLC-UV followed by acidification with phosphoric acid (0.1%) and extraction with ethyl acetate. Dried samples were dissolved in methanol–water, (1:1, v/v) plus 0.1% formic acid, and 20 μL injected per run with a Waters autosampler.

Phenolics isolated by HPLC-UV were dissolved in 0.8 ml of CD_3OD, and ^{1}H-NMR spectra were acquired deuterium locked at 25 °C using a Bruker QE 300 MHz NMR spectrometer. Chemical shift values were assigned relative to the frequencies of residual nondeuterated water and methanol externally referenced to tetramethylsilane (TMS).

1.6. Extracellular antioxidant assay

The extracellular antioxidant capacity was estimated using a chemiluminescent assay that determined the quantity of H_2O_2 consumed by samples [5]. Samples (0.4 ml) of treated or untreated suspension cells were dispensed into tubes, and placed into an EG and G Berthold Autolumat 953 luminometer (Bad Wildbad, Germany). Two stock solutions were prepared: (A) 0.5 mM H_2O_2 in the same assay buffer used for cell suspensions; and (B) horseradish peroxidase, 28.8 U/ml, and 1.71 mM luminol in 1 M $NaPO_4$, pH 7. The luminometer first added stock solution A (50 μL) followed 4.5 s later by stock solution B (50 μL). The final concentrations were 50 μM H_2O_2, 1.44 U/ml of peroxidase and 171 μM luminol. Chemiluminescence was measured as relative light units (RLU) every 0.1 s for 20 s; the maximum measurement is proportional to the H_2O_2 concentration. Standard curves were prepared with dilutions of H_2O_2 in assay buffer. Under these assay conditions, the extracellular antioxidant in each sample had sufficient time to react with the added H_2O_2; the remaining H_2O_2 reacted with luminol. The decrease in RLU in suspension samples compared to buffer controls corresponds to the H_2O_2 consumed by extracellular antioxidant in each sample and provides an estimate of the extracellular antioxidant concentration of each sample.

2. Results

2.1. Identification extracellular phenolics

Eight extracellular phenolic compounds from *N. tabacum* cells were separated by C_{18}-HPLC and identified on the basis of their HPLC elution times, UV absorbance spectra, ES^--MS mass spectra, and in some cases proton NMR spectra (Table 1). The phenolics belonged to two classes: four were hydroxycinnamic acid amides and four were derivatives of acetophenone. The elution times, UV spectra, and ES^--MS spectra of peaks 1 and 2 (Table 1) were identical to those of authentic standards of *N*-(*E*)-caffeoylputrescine ($C_{13}H_{18}N_2O_3 = 250$; Fig. 1, structure **1**) and *N*-(*E*)-feruloylputrescine ($C_{14}H_{20}N_2O_3 = 264$; Fig. 1, structure **2**), respectively. The elution time, UV spectrum, ES^--MS spectrum, and ^{1}H-NMR spectrum of peak 8 were identical to those of an authentic standard of *N*-(*E*)-feruloyltyramine ($C_{18}H_{19}NO_4 = 313$; Fig. 1, structure **8**). ES^--MS spectra of peaks 7 and 8 were indistinguishable, but the UV spectra were markedly different, suggesting that peak 7 is the *cis* isomer of peak 8, *N*-(*Z*)-feruloyltyramine ($C_{18}H_{19}NO_4 = 313$; Fig. 1, structure **7**). This was confirmed by the ^{1}H-NMR signal for the olefinic proton on C8, a doublet with $J = 12.5$ Hz at 5.80 ppm, indicative of a *cis* double bond between C7 and C8 [21].

Nicotiana tabacum phenolics in HPLC peaks 3, 4, 5, and 6 were identified as acetophenone derivatives (Table 1 and Fig. 1). The elution times, UV spectra, and ES^--MS spectra of peaks 5 and 6 were identical to those of authentic standards of 4-hydroxyacetophenone ($C_8H_8O_2 = 136$; Fig. 1, structure **5**) and 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone; $C_{10}H_{12}O_4 = 196$; Fig. 1, structure **6**), respectively. In addition, the ES^--MS and ^{1}H-NMR spectra obtained for peak 6 were in accord with those recently reported for acetosyringone [9]. The UV spectra of peaks

Table 1
Hydroxycinnamic acid amide conjugates and acetophenone derivatives released from cultured cells of *N. tabacum* and quantified by HPLC-UV analysis

	Elution time (min)	UV Abs. maxima (210–330 nm)	ES^--MS ions		Identification
			$[M-1]^-$	100% RI[a]	
Hydroxycinnamic acid amides					
Peak 1	9.6	319, 294 (sh)	249	249	*N*-(*E*)-caffeoylputrescine
Peak 2	13.8	318, 294 (sh)	263	263	*N*-(*E*)-feruloylputrescine
Peak 7	28.5	276, 305 (sh)	312	312	*N*-(*Z*)-feruloyltyramine
Peak 8	30.9	318, 293 (sh)	312	312	*N*-(*E*)-feruloyltyramine
Acetophenone derivatives					
Peak 3	20.2	301, 215	211	196	α-Hydroxyacetosyringone
Peak 4	22.4	299, 215	181	166	5-Hydroxyacetovanillone[b]
Peak 5	24.7	276, 220	135	135	4-Hydroxyacetophenone
Peak 6	25.9	300, 215	195	180	Acetosyringone

[a] RI, relative intensity.
[b] Tentative identification based on HPLC, LC-MS, and UV absorbance data.

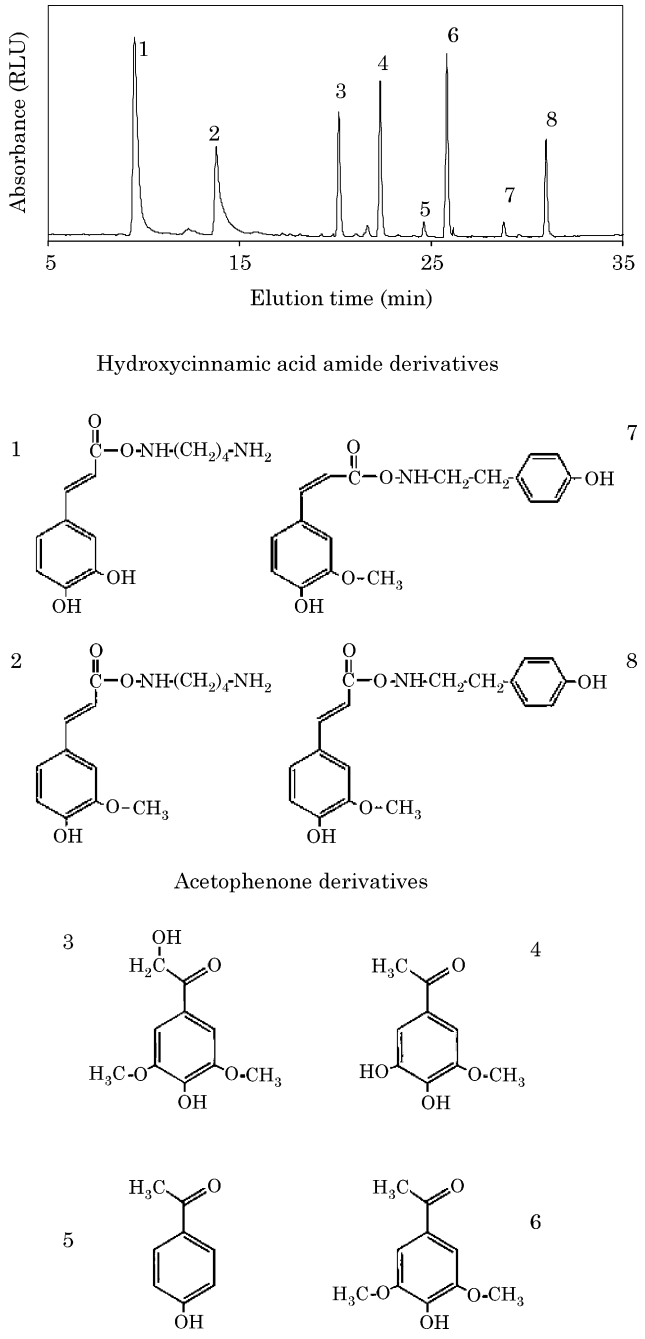

Fig. 1. HPLC-UV chromatogram and structures of extracellular phenolics that accumulate in tobacco cell suspensions. The chromatogram is a composite of several samples in order to include all of the phenolics in a single chromatogram. The numbering of the hydroxycinnamic acid amide conjugates and acetophenone derivatives corresponds to that of the HPLC peaks on the chromatogram as well as Table 1: 1, *N*-(*E*)-caffeoylputrescine; 2, *N*-(*E*)-feruloylputrescine; 3, α-hydroxyacetosyringone; 4, 5-hydroxyacetovanillone; 5, 4-hydroxyacetophenone; 6, acetosyringone; 7 and 8, *Z* and *E* isomers, respectively, of *N*-feruloyltyramine.

3 and 4 were nearly identical to that of acetosyringone (peak 6), whereas the molecular ion minus a proton, $[M-1]^-$, was at *m/z* 211 for peak 3, *m/z* 181 for peak 4, and *m/z* 195 for peak 6. These data indicate that peak 3 differs from acetosyringone by the addition of an oxygen atom to the alpha carbon and peak 4 differs from acetosyringone by the loss of a methyl group from either of the symmetric methoxy groups on the phenyl ring. Hence, peak 3 was identified as α-hydroxyacetosyringone ($C_{10}H_{12}O_5=212$; Fig. 1, structure **3**) and peak 4 was tentatively identified as 3-methoxy-4,5-dihydroxyacetophenone (5-hydroxyacetovanillone; $C_9H_{10}O_4=182$; Fig. 1, structure **4**). Peaks 5 (4-hydroxyacetophenone) and 7 (*N*-(*Z*)-feruloyltyramine) were much less abundant than the other six peaks and are therefore not mentioned further in the results.

2.2. Phenolic accumulation in suspensions treated with *P. syringae* isolates

The response of tobacco cell suspensions, 0.05 g ml^{-1}, to *P. syringae* strains WT (HR+) or B7 (HR−), 10^7 cfu ml^{-1}, was followed over a 10†12 h period (Fig. 2). The accumulation of extracellular phenolics was monitored by HPLC-UV analysis of the extracellular fluid.

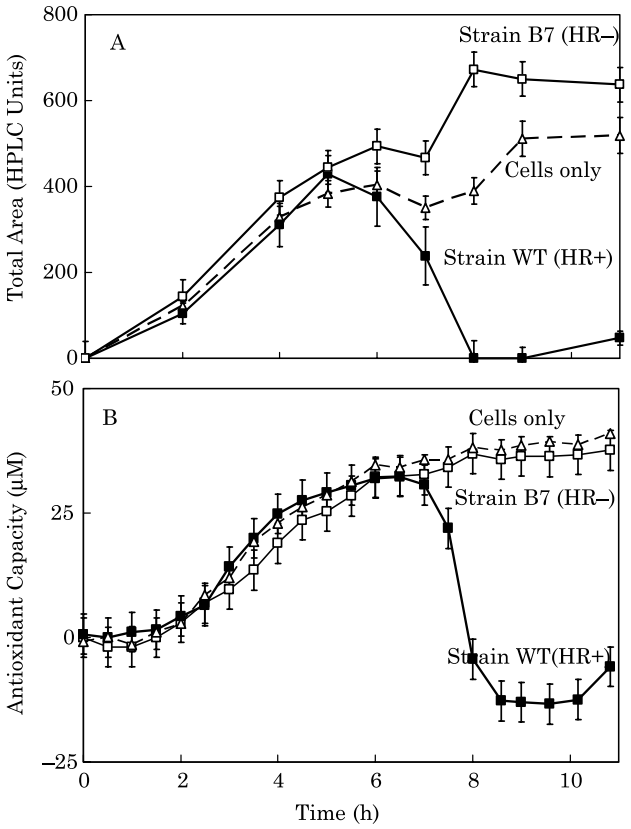

Fig. 2. Time course monitoring changes in extracellular phenolic accumulation and antioxidant capacity. Two-day-old tobacco cell suspensions, 0.05 g ml^{-1}, were treated with *P. syringae* strains, 10^7 cfu ml^{-1}. Samples of treated tobacco cell suspensions were removed and analyzed: (A) total peak area from HPLC-UV chromatogram; (B) antioxidant capacity. See Section 2 for details. The data shown represent the results of one experiment with two replicates of each treatment. The experiment was repeated two additional times with similar results. The error bars represents 1 SD.

At this inoculum concentration, the extracellular phenolics in tobacco suspensions increased at about the same rate in both control and bacteria-treated cell suspensions during the first 4–5 h (Fig. 2A). The extracellular phenolics in control and strain B7 (HR−) treated suspensions continued to increase, except for a slight dip at 7 h in peak area, and reached a maximum after 8–9 h. Conversely, the extracellular phenolics of strain WT (HR+) treated suspensions began to decrease rapidly after 6 h. There were no detectable HPLC peaks in the WT (HR+) treated suspensions at 8 h, however, by 11 h extracellular phenolics began to reappear (Fig. 2A).

The loss of extracellular phenolics in tobacco cell suspensions treated with strain WT (HR+) was concurrent with the onset of an oxidative burst in these cells. This was determined by monitoring the extracellular antioxidant capacity of these suspensions by titration with H_2O_2 (Fig. 2B). Control and B7 treated suspensions increased in antioxidant capacity, nearly 40 μM after 10 h, as the concentration of extracellular phenolics increased. The WT (HR+) treated suspensions increased in antioxidant capacity to about 35 μM by 6 h after which there was a sudden decrease in extracellular antioxidant capacity to −15 μM. This decrease in antioxidant capacity is indicative of an oxidative burst which appears to be over by 11 h when antioxidant capacity starts to increase again. The extracellular prooxidants produced, such as H_2O_2, oxidize the available phenolics and any excess prooxidant is indicated as negative antioxidant capacity in this assay [5].

The phenolic compositions of the different treatments were compared after 9 h (Fig. 3A). Five major phenolics were identified in the extracellular fluid of cell suspensions from 2-day-old tobacco cultures. Two of the phenolics were conjugates of putrescine and three were acetophenone derivatives. The phenolic compositions of the control and strain B7 (HR-) treatments were qualitatively similar, but the latter was generally 10–20% higher in concentration. As a result of the oxidative burst, the strain WT (HR+) treatment had no detectable extracellular phenolics after 9 h.

The changes in the concentration of individual phenolics during the 11 h incubation period generally followed one of three patterns in the control and strain B7 (HR−) treatments (Fig. 3B). The two putrescine conjugates increased immediately reaching maximum levels by 4–5 h and then after a slight decrease stabilized over the remainder of the monitored period as shown for caffeoylputrescine. The three acetophenone derivatives increased after nearly a 2 h delay and then followed two different patterns. The 5-hydroxyacetovanillone derivative reached a maximum at about 5 h and then decreased to undetectable levels by 9 h in control suspension cells; in strain B7 (HR−) treatments it decreased but was still detectable. The other acetophenone derivatives increased at a nearly constant rate for the remainder of the 11 h period as shown for acetosyringone. The decrease in the putrescine conjugates and 5-hydroxyacetovanillone accounts for the above-mentioned dip in total peak area at 6–7 h in the control and strain B7 (HR−) treatments (Fig. 2A).

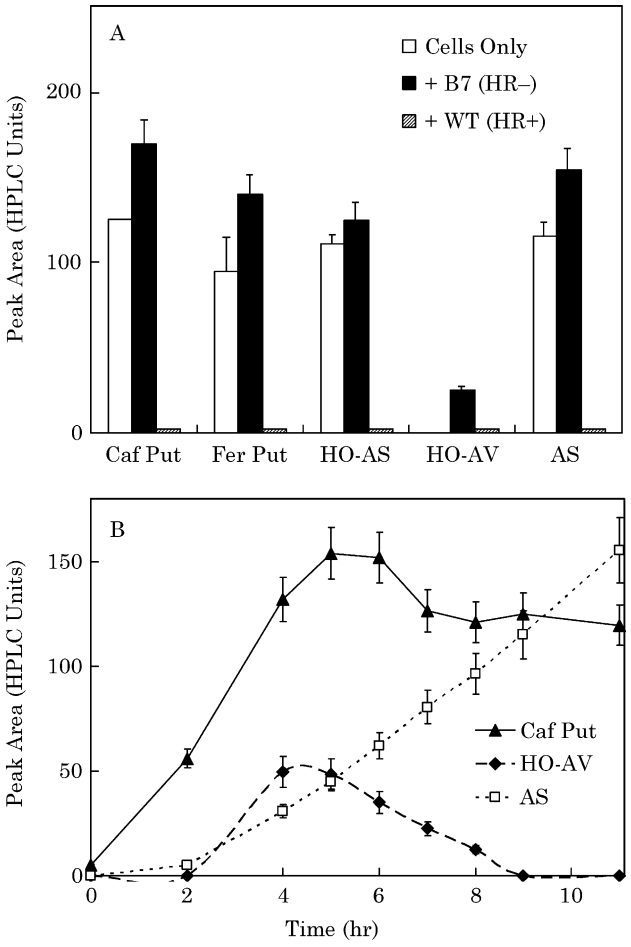

Fig. 3. Major extracellular phenolics in cell suspensions from 2-day-old tobacco cultures, 0.05 g ml^{-1}, treated with *P. syringae* strains, 10^7 cfu ml^{-1}. (A) Major extracellular phenolics after 9 h of incubation, based on HPLC-UV analysis. (B) Different patterns of accumulation of phenolics in the extracellular fluid of untreated tobacco cell suspensions. See Section 2 for details. The data shown represent the results of one experiment with two replicates of each treatment. The experiment was repeated two additional times with similar results. The error bars represents 1 SD. (Caf Put, caffeoylputrescine; Fer Put, feruloylputrescine; HO-AS, α-hydroxyacetosyringone; HO-AV, 5-hydroxyacetovanillone; AS, acetosyringone).

2.3. Effect of bacterial inoculum concentration on extracellular phenolic accumulation

Previous experience with this tobacco cell suspension model system has shown that varying the bacterial inoculum concentration will strongly influence many responses associated with the plant–bacterial interaction [3,6]. Therefore we examined the effect of bacterial inoculum concentration on the accumulation of extracellular phenolics over a 6 h period using cells from 2-day-old tobacco cultures (Fig. 4A). Only strain B7 (HR−) was used since it induces

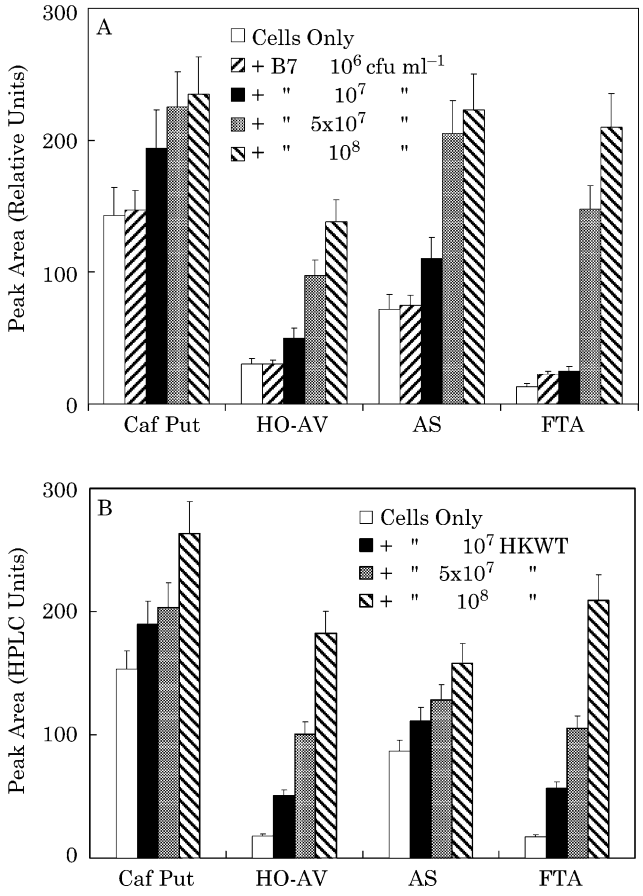

Fig. 4. Effect of inoculum concentration of live or heat-killed bacteria on the composition of extracellular phenolics in tobacco cell suspensions. Two-day-old tobacco cell suspensions, 0.05 g ml^{-1}, were treated with designated concentrations of (A) strain B7 (HR−) or (B) heat-killed preparations (HKWT) of strain WT (HR+) for 6 h. See Section 2 for details. The data shown represent the results of two experiments, each with two replicates of each treatment. The error bars represents 1 SD. (Caf Put, caffeoylputrescine; HO-AV, 5-hydroxyacetovanillone; AS, acetosyringone; FTA, feruloyltyramine).

accumulation of phenolics without production of the oxidative burst. As the inoculum concentration increased from 10^6 to 10^8 cfu ml^{-1}, the concentration of many of the extracellular phenolics increased in an incremental manner similar to caffeoylputrescine with an overall increase of about 50% at the highest inoculum concentration of 10^8 cfu ml^{-1}. The concentration of three phenols, hydroxyacetovanillone, acetosyringone and feruloyltyramine, responded more dramatically increasing 3-, 4- and 8-fold, respectively, as the inoculum concentration increased from 10^6 to 10^8 cfu ml^{-1}.

To see if live bacteria were required for the increased accumulation of extracellular phenolics, we treated tobacco suspensions with heat-killed *P. syringae* strain WT (HR+) equivalent to 10^7, 5×10^7, and 10^8 cfu ml^{-1} (Fig. 4B). In general, the response after 6 h incubation was similar to that associated with live bacteria. The concentration of extracellular phenolics in tobacco suspensions increased as the concentration of the heat-killed bacteria treatment increased. The most dramatic change in concentration occurred with hydroxyacetovanillone and feruloyltyramine, which increased 9- to 10-fold at the highest concentration of heat-killed bacteria compared to untreated tobacco cells.

2.4. Effect of tobacco cell age and density on extracellular phenolic accumulation

During attempts to accumulate large quantities of these phenolics for identification, we found that increased tobacco suspension cell density did not always yield increased phenolics. Tobacco cell suspensions of density 0.05, 0.1 and 0.2 g ml^{-1}, were incubated 6 h with strain B7 (HR−), 10^7 cfu ml^{-1} (Fig. 5). Based on the total peak area, when the suspension cell density was doubled to 0.1 g ml^{-1} the extracellular phenolics increased only 34%. Increasing the cell density further to 0.2 g ml^{-1} surprisingly, caused the concentration of total phenolics to drop

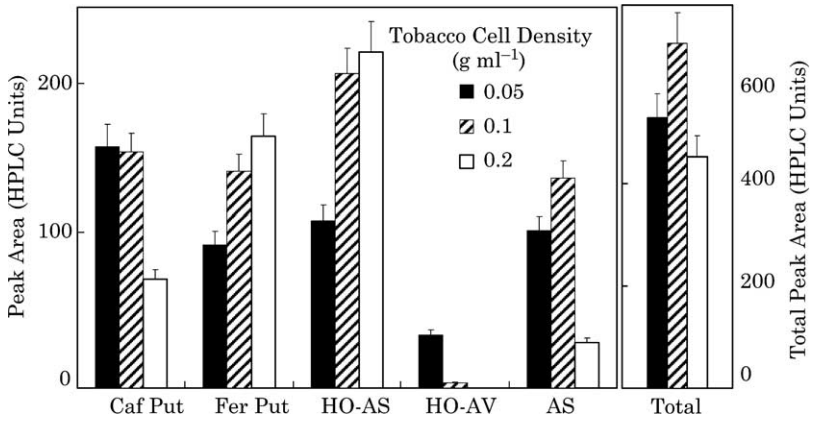

Fig. 5. Effect of tobacco suspension cell density on the composition of extracellular phenolics. Two-day-old tobacco cell suspensions of different cell densities were treated with *P. syringae* strain, 10^7 cfu ml^{-1}, for 6 h. Only phenolics that changed substantially with cell density are shown. The total peak area includes all phenolics, shown and not shown in the figure. See Section 2 for details. The data shown represent the results of two experiments, each with two replicates of each treatment. The error bars represents 1 SD. (Caf Put, caffeoylputrescine; Fer Put, feruloylputrescine; HO-AS, α-hydroxyacetosyringone; HO-AV, 5-hydroxyacetovanillone; AS, acetosyringone).

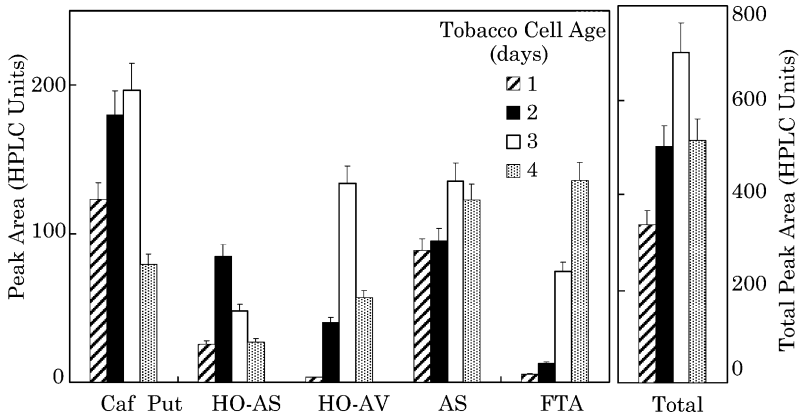

Fig. 6. Effect of tobacco cell age on the composition of extracellular phenolics. Tobacco cell suspensions were prepared from 1- to 4-day-old tobacco cultures, 0.05 g ml^{-1}, and treated with *P. syringae* strain, 10^7 cfu ml^{-1}, for 6 h. Only phenolics that changed substantially with cell age are shown. The total peak area includes all phenolics, shown and not shown in the figure. See Section 2 for details. The data shown represent the results of two experiments, each with two replicates of each treatment. The error bars represents 1 SD. (Caf Put, caffeoylputrescine; HO-AS, α-hydroxyacetosyringone; HO-AV, 5-hydroxyacetovanillone; AS, acetosyringone; FTA, feruloyltyramine).

below levels in the 0.05 g ml^{-1} density. The individual phenolics did not necessarily follow this pattern (Fig. 5). For example, the concentration of feruloylputrescine and α-hydroxyacetosyringone increased with cell density, whereas the concentration of caffeoylputrescine decreased with increased cell density. The concentration of acetosyringone decreased more than 70% at the higher cell density.

Previous experience with these suspension cells has indicated that cell age is critical to obtain the expected differential responses with the *P. syringae* pathovars and strains; therefore we determined the effect of cell age on extracellular phenolic accumulation after 6 h incubation (Fig. 6). To simplify the figure only phenolics greatly affected by cell age were included. Some of the dominant phenolics that accumulate in cell suspensions from 2-day-old tobacco cultures do not maintain these levels in cell suspensions from 4-day-old tobacco cultures, for example caffeoylputrescine and α-hydroxyacetosyringone. Other phenolics, such as 5-hydroxyacetovanillone and feruloyltyramine, which is normally a minor component, accumulate to higher levels in 3- or 4-day-old cells and become the dominant phenolics.

3. Discussion

This study characterized the accumulation of extracellular phenolics in tobacco cell suspensions after treatment with a bacterial pathogen. Two groups of phenolics were identified: the acetophenone group consisted of hydroxyacetosyringone, hydroxyacetovanillone, hydroxyacetophenone, and acetosyringone; and the hydroxycinnamic acid amide group consisted of caffeoylputrescine, feruloylputrescine, and feruloyltyramine (Table 1, Fig. 1). Most of the extracellular phenolics were found to accumulate in untreated tobacco cell suspensions, however, the rate and magnitude of accumulation as well as different phenolics increased in the presence of *P. syringae* strain B7 (HR−) (Fig. 4). The rate of accumulation and concentration of the extracellular phenolics in tobacco cell suspensions increased with higher inoculum concentrations of either live or heat-killed bacteria. Other factors that affected the accumulation of extracellular phenolics in tobacco cell suspensions were the tobacco cell density and age. Interestingly, doubling the cell density of tobacco suspensions from 0.1 to 0.2 g ml^{-1} resulted in a decrease in accumulation rate and concentration of extracellular phenolics (Fig. 5). The age of the tobacco cells affected the qualitative and quantitiative makeup of the extracellular phenolics, with the relatively minor component, feruloyltyramine, increasing dramatically as cells age (Fig. 6). When tobacco cell suspensions were treated with *P. syringae* strain WT (HR+), the extracellular phenolics started to accumulate similarly to strain B7 (HR−) treatments until the onset of an oxidative burst (Fig. 2). During the oxidative burst, the concentration of the extracellular phenolics in these tobacco suspensions decreased and became undetectable.

The phenolics identified in this study fall into two groups of metabolites, the acetophenones and hydroxycinnamic acid amides, that are associated with bioactivity in both plant and animal cells as established by several recent reviews [8,11,24,31]. The acetophenone derivatives (Fig. 1) have been linked to regulation of critical gene expression and metabolite production in several host–pathogen interactions [12,19]. We recently reported that increasing the concentration of extracellular acetosyringone in tobacco cell suspensions treated with *P. syringae* WT (HR+) caused an oxygen uptake response, unique to incompatible interactions, to occur as much as 1.5 h earlier. The hydroxycinnamic acid amides, which include the conjugated putrescine and tyramine derivatives (Fig. 1B) have properties associated with polyamines, which are derived from arginine and are positively charged at physiological pH. Polyamines are ubiquitous in nature and are known to bind

to negatively charged molecules such as nucleic acids and cell walls [18]. Although their production and presence is strongly tied to several physiological processes, their specific putative roles remain controversial [11,31].

Numerous studies have demonstrated that periods of oxidative stress occur during the course of plant–pathogen interactions [1]. As shown in this study (Fig. 2), this event would oxidize redox-sensitive phenolics, affecting their concentration as well as any associated bioactivity which could influence the outcome of the interaction. There are several studies relating redox sensitivity to bioactivity in phenols. A strong correlation has been found between redox potentials (E^o) and the inhibitory effects of naphthoquinones on Epstein-Barr virus activation, which could lead to cancer [13]. The more positive the redox potential the stronger the anti-tumor promoting effect. They point out that changes in pH would alter the redox potential and therefore alter the bioactivity of the phenolic [28]. Loo [17] in a recent review discussed several redox-sensitive mechanisms in which plant phenolics are used as chemopreventive agents to prevent cancer cell proliferation. The sites include redox-sensitive regulation of kinases as well as transcription factors. These studies also point out the fact that phenolics when oxidized give up an electron and can become strong oxidants which in the right location could interact with regulatory molecules and trigger or suppress a particular mechanism.

In summary, this study demonstrated that plant suspension cells actively and rapidly accumulate phenolics in the extracellular environment during the first several hours of the plant–bacterial interaction. Preliminary experiments examining phenolics from extracellular fluids of leaves suggest similar events may occur in planta. Because these phenolics are in direct contact with the bacterial pathogens and because of their reported bioactivity [12,16,20,26,30], it is reasonable to speculate that they play a pivotal role in pathogenesis. Our goal now is to determine whether these extracellular phenolics have bioactive properties that may play a significant role in our cell-suspension–bacterial interaction.

Acknowledgements

We wish to thank Dr Walter F. Schmidt and Mrs Ute Klingebiel, USDA, ARS, Environmental Quality Laboratory for acquisition of the ^{1}H-NMR spectra.

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